Vol. 7, 817–821, September 1998 

RWLP0074

***Short Communication***

# 4-Hydroxy-1-(3-pyridyl)-1-butanone-Hemoglobin Adducts as Biomarkers of Exposure to Tobacco Smoke: Validation of a Method to Be Used in Multicenter Studies[1]

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## Abstract

**Hemoglobin (Hb) adducts of 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB), a metabolite of two tobacco-specific nitrosamines [4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and *N'*-nitrosonornicotine], were measured as biomarkers of exposure to tobacco smoke as part of a study on genetic alterations and susceptibility to lung cancer among nonsmokers. HPB-Hb adducts were measured after collection of RBCs by Ficoll gradient in six collaborating centers, release of HPB by alkaline hydrolysis from Hb, clean-up by solid-phase extraction, and analysis of an electron-capturing derivative by gas chromatography-electron capture mass spectrometry. Prior to analysis of samples from study subjects, the reproducibility of this approach was validated in blood from donors. The coefficient of variation of reproducibility of paired aliquots from five samples ranged from 7 to 25%; the within-sample reproducibilities of four and eight aliquots were 4 and 16%, respectively. The study subjects consisted of 18 smokers and 52 never-smokers. HPB-Hb adduct levels were significantly higher ($P = 0.02$) in smokers ($26 \pm 13$ fmol HPB/g Hb) than in never-smokers ($20 \pm 8$ fmol HPB/g Hb). There was no difference between sexes. These results suggest that the level of HPB-Hb adducts, measured using a method modified to facilitate use in multicenter studies, can be a useful biomarker of exposure to tobacco smoke.**

## Introduction

Tobacco smoking is an important causative factor in cancer of the lung, as well as in cancers of other organs, such as larynx, oral cavity, pancreas, and bladder (1). Although most individuals who develop lung cancer are smokers, 2–10% are non-smokers (2). A methodological problem in the study of exposure to tobacco smoke is the lack of a sensitive and reliable biomarker for the biologically effective dose. A possible solution to this problem is the application of analytical methods that can measure the extent to which a tobacco-specific carcinogen binds to biomolecules such as DNA or proteins. The analytical method applied in this study measures the level of adducts with Hb[5] of HPB, a common metabolite of two tobacco-specific nitrosamines, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and *N'*-nitrosonornicotine. Although the persistence of HPB-Hb adducts has not been adequately determined in humans, studies have estimated that Hb adducts of the aromatic amine 4-aminobiphenyl, also formed as a result of exposure to tobacco smoke, have a half-life in humans of 7–9 weeks (3, 4). Thus, the concentration of HPB released from Hb should give an indication of the amount of tobacco-specific nitrosamines to which an individual has been exposed during the few months prior to examination.

The purpose of this study was to test whether a method for measuring HPB-Hb adducts, first described by Carmella and coworkers (5), could be modified to facilitate multicenter sample collection and to simplify sample clean-up. The modified method involved parallel collection of RBCs on a Ficoll gradient in six centers and shipment to a central laboratory for analysis, alkaline hydrolysis of HPB from Hb, purification of HPB by solid-phase extraction, and formation of an electron-capturing derivative that was purified by solvent extraction prior to analysis by gas chromatography-electron capture mass spectrometry. This report describes the validation of this modified method to assess exposure to tobacco smoke.

## Materials and Methods

**Materials.** Reagents and solvents were obtained from commercial sources in ultrapure (+99.9%) or HPLC grades (Fluka

Received 9/22/97; revised 5/27/98; accepted 6/11/98.

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[1] This study was partially supported by European Commission Grant DG-XII (Environment Programme, Contract EV5V-CT94-0555). The work reported in this paper was undertaken during the tenure of IARC Special Training Awards (to S. E. A. and S. L.).

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[5] The abbreviations used are: Hb, hemoglobin; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone; HPLC, high-performance liquid chromatography; GC/MS, gas chromatography-mass spectroscopy; % CV, percentage coefficient of variation.

Chemie AG, Buchs, Switzerland; SDS, Peypin, France; or E. Merck, Darmstadt, Germany). [4,4-$d_2$]-HPB and nonlabeled HPB standards were a generous gift from Stephen S. Hecht (University of Minnesota Cancer Center, Minneapolis, MN). The HPB-$d_2$ standard had an isotopic purity of 98.3%, which was corrected for by the quantification procedure used.

Isotonic buffer was prepared by mixing 1 volume of solution A with 9 volumes of solution B. Solution A was prepared by dissolving 1.0 g of glucose, 0.0074 g of $CaCl_2$, 0.1992 g of $MgCl_2$, 0.4026 g of KCl, and 17.565 g of Tris in 950 ml of distilled water, adjusting the solution to pH 7.6 with HCl, and increasing the volume to 1 liter with distilled water. Solution B was solution of 8.19 g of NaCl in 1 liter of distilled water.

**Blood Samples.** Blood samples for method validation were obtained from the Center de Transfusion Sanguine (Lyon, France). Blood samples were collected into 7-ml blood collection tubes containing citrate anticoagulant. Blood was stored at +4°C until analysis, which was carried out the day following collection, using the method outlined below.

An additional set of study samples was obtained from subjects recruited within the framework of a multicenter study on genetic alterations and susceptibility to lung cancer among nonsmokers. These subjects were patients admitted to hospital with a diagnosis of neoplastic or nonneoplastic disease or healthy subjects randomly selected from the general population. A total of 70 subjects were studied, including 14 men and 56 women. Eighteen were smokers and 52 were never-smokers, *i.e.*, they had never smoked more than one cigarette daily for 1 year ( or the equivalent in other tobacco products).

**Blood Collection and Isolation of Hb.** To permit simultaneous analysis of carcinogen-protein adduct levels, genetic polymorphisms of carcinogen-metabolizing enzymes and DNA repair enzyme activities, it was necessary to separate blood samples into plasma, WBCs, and RBCs before storage and shipment to IARC for analysis.

Fifteen-ml samples of blood, taken by venipuncture, were collected into three 5-ml or one 15-ml blood collection tube containing citrate anticoagulant. The combined sample was diluted by mixing with an equal volume of isotonic buffer in a sterile 50-ml polypropylene tube. This dilution controls the degree of aggregation of the RBCs so that they sediment easily, with a minimum of lymphocyte entrapment. Once diluted, the sample was processed immediately.

The 30 ml of diluted blood were then poured on top of the plastic insert of a UNI-SEP Maxi Ficoll density gradient centrifugation tube (Wak-Chemie Medical Gmbh, Bad Homburg, Germany). These tubes contain a solution of 5.6% polysucrose and 9.6% sodium metrizoate, which is held in the bottom of the tubes by a one-way plastic porous barrier. After the tubes were capped and centrifuged (at 18–20°C) for 20 min at 1000 × *g*, aggregated RBCs and granulocytes sedimented to the bottom of the tubes, whereas the WBC migrated to the interface between the plasma and the polysucrose-sodium metrizoate layers, commonly referred to as the Ficoll layer.

After centrifugation, plasma and WBCs were carefully removed, processed, and stored for other analysis. The polysucrose-sodium metrizoate layer above the porous barrier was then removed with a Pasteur pipette and discarded. Using a clean tweezers, the porous plastic barrier was carefully removed and discarded. The polysucrose-sodium metrizoate layer above the RBCs in the bottom of the tube was also removed and discarded using a Pasteur pipette, and the technicians were careful not to disturb the RBC layer at the bottom of the tube. The RBCs were transferred to a centrifuge tube containing at least 3 times the volume of isotonic buffer. The RBCs were suspended evenly and centrifuged at 1000 × *g* for 10 min. The supernatant was removed and discarded, and the washing procedure was repeated. The washed RBCs were then transferred into a 4.5-ml Nunc cryotube (Nunc/AS, Kamstrup, Denmark), and a small amount (<0.5 ml) of isotonic buffer was added, if necessary. The RBC solutions were stored at −80°C until shipment to IARC for analyses.

**Isolation of Hb.** At IARC, washed RBCs were lysed by the addition of 3 volumes of ice-cold HPLC-grade water (BDH, Poole, England). After thorough mixing by vortex for 5 min, the phosphate concentration of the solution was adjusted to 0.2 M with cold 0.67 M potassium phosphate buffer (pH 6.5) and was left to stand for 15 min. Red cell membranes were removed by centrifugation at 18,000 rpm for 30 min. The Hb was dialyzed for 48 h against 20–50 volumes of distilled water using Spectra/Por 4 cellulose dialysis tubing, with a molecular weight cutoff of $M_r$ 12,000–15,000 (Poly Labo Block, Strasbourg, France). The water was changed every 12 h (5). The Hb content of the dialysate was determined with Drabkin's Reagent (Sigma Chemical Co., St. Quentin Fallavier, France).

Duplicate 7-ml fractions of the dialyzed Hb solution, each corresponding to 4–5 ml of blood, were aliquoted into 30-ml Pyrex glass tubes (26-mm external diameter × 100 mm), with Teflon-sealed caps, for storage at −80°C until HPB analysis. The remaining dialyzed Hb solution was aliquoted into two 4.5-ml Nunc cryotubes and was stored at −80°C. These solutions are available for analysis of additional biomarkers.

**Release of HPB from Hb and Clean-up by Solid-phase Extraction.** The Hb solution stored in the Pyrex glass storage tubes was allowed to thaw, and 100 pg of HPB-$d_2$ in 20 μl of water were added as internal standard. To release HPB, the concentration of the solution was brought to 0.11 M NaOH by addition of 1 M NaOH. The mixture was sonically dispersed for 1 h, and proteins were precipitated with 14–20% (v/v) of 20% $(NH_4)_2SO_4$. Precipitated protein was removed by centrifugation at 2600 rpm for 10 min at 15°C.

The clear supernatant was applied to a 6-ml Extract-Clean solid-phase extraction column (Altech Associates, Deerfield, IL) containing 500 mg of C18 packing. The column had been preconditioned by washing with 3–5 ml of HPLC-grade methanol, 5 ml of water, and 5 ml of 0.067 M $KH_2PO_4$ buffer (pH 6.7). The solution was allowed to drain through the column under gravity; columns were not allowed to run dry. The HPB on the column was then washed with 5 ml of HPLC-grade water or 0.001 M $KH_2PO_4$, which had been adjusted to pH ~7.0 with 0.05 N NaOH or 0.05 N $H_3PO_4$. (Recoveries were inconsistent if wash solution was not buffered to pH ~7.) Following the wash, the column was drained well, and HPB was eluted under gravity with 2 ml of methanol into a 2-ml disposable glass vial (Wheaton, Millville, NJ). Methanol remaining on the column was pulled into the tube under vacuum (~3 mmHg). The methanol eluate was taken to dryness at room temperature overnight in a SpeedVac concentrator. Recovery of HPB for this step was determined to be ~80%.

**Derivatization of HPB.** One ml of $CH_2Cl_2$, 1 ml of trimethylamine hydrochloride solution in hexane, prepared as described previously (5), and 10 μl of a 10% solution of pentafluorobenzoylchloride in hexane were added to the glass vial containing the dried HPB residue. After mixing, the solution was allowed to stand at room temperature for 2 h and then taken to dryness with a SpeedVac concentrator.

To remove unreacted pentafluorobenzoylchloride and traces of Hb, the derivatized HPB solution was made acidic

with 100 $\mu$l 1 N HCl and extracted twice with 1 ml of hexane for 1.5 min. After centrifugation in the SpeedVac for 5 min (no vacuum applied), the hexane layer was discarded, and the hexane clean-up was repeated. The pH of the aqueous layer was then raised to 7.1 by addition of sufficient 0.5 M $Na_2PO_4$ (pH 9). HPB-pentafluorobenzoate was then extracted twice with 0.9 ml of hexane with 1.5 min of mixing. The combined hexane layers were transferred to a 1.5-ml disposable glass vial (Chromacol Ltd., Herts, United Kingdom), brought to dryness on SpeedVac, and stored at −20°C until GC/MS analysis. The recovery of HPB-pentafluorobenzoate was determined to be ~80%.

**GC/MS.** The purified HPB-pentafluorobenzoate derivative was taken up in 9 $\mu$l of isopropanol, and 3-$\mu$l aliquots were analyzed by gas chromatography-negative ion chemical ionization mass spectrometry by selected ion monitoring. GC/MS was carried out on a Hewlett Packard 5980A gas chromatograph equipped with a splitless injector (270°C) coupled to a Hewlett Packard 5988A mass spectrometer. The analysis was carried out on a slightly polar DB1701 fused silica capillary column (30 m × 0.25 mm inner diameter; film thickness, 0.33 $\mu$m), coupled directly to the mass spectrometer ion source (150°C) through a heated interface (270°C). Helium was used as carrier gas at a column head pressure of 150 kPa. The temperature of the gas chromatography oven was raised from an initial temperature of 100°C to 280°C at 20°C/min, where it was held for 2 min. Under these conditions, the retention time of the pentafluorobenzoate derivative of HPB was ~9 min.

The mass spectrometer was operated in the negative ion mode with methane as reagent gas (~1 torr). The mass spectrometer was tuned to monitor negative molecular ions at *m/z* 358.9 (HPB-$d_0$-pentafluorobenzoate)$^-$ and *m/z* 360.9 (HPB-$d_2$-pentafluorobenzoate)$^-$. The detection limit for HPB was 1 pg HPB/injection.

Identification of a chromatographic peak as HPB was dependent on it retention time being ~0.007 min greater than that of the HPB-$d_2$ internal standard. Amounts of HPB in each sample were calculated from a plot of the ratio of the integrated peak areas of *m/z* 358.9 and *m/z* 360.9 on the *Y* axis *versus* the amount of HPB on the *X* axis. A linear calibration curve was obtained over the range of 2–100 pg per sample. Only results above a cutoff point of 2 pg/sample were reported as detected. This corresponds to an approximate doubling of the area of the background peak resulting from the isotopic impurity of 1.7 pg HPB-$d_0$/100 pg HPB-$d_2$, which was observed when the HPB-$d_2$ internal standard was injected. The amount of HPB released from a particular sample of Hb was expressed as the amount of HPB (in fmol) divided by the amount of Hb in the sample (in g). Fig. 1 shows the selected ion mass chromatogram for HPB-$d_0$ (*m/z* 358.9) and HPB-$d_2$ (*m/z* 360.9) for a Hb sample that was found to release 44 fmol HPB/g Hb.

**Statistical Analysis.** For 52 subjects for whom duplicate samples were analyzed, the mean of the two values was recorded as the subject's HPB level for purposes of grouped analysis. For seven subjects whose HPB-Hb adduct levels were too low to be determined, the value of the detection limit for that sample was included in statistical analysis. The mean and SD of HPB values were calculated for groups of study subjects defined according to sex and smoking status. Because the distribution of HPB-Hb adduct level values was not normal, we used the nonparametric Kruskal-Wallis test of rank sum, and we repeated the analysis after log transformation.

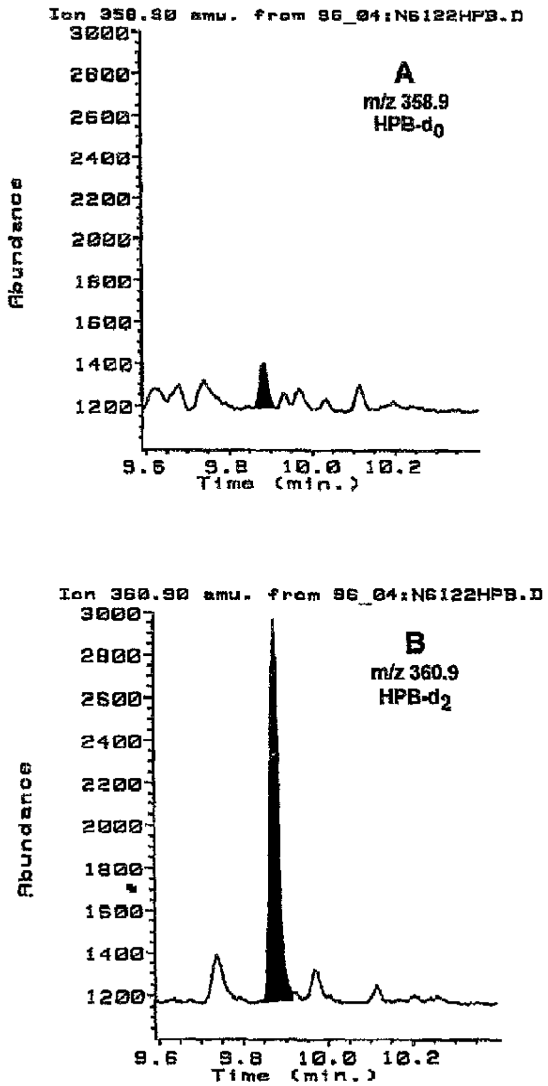


*Fig. 1.* Selected ion monitoring mass chromatograms for sample 25208, found to contain 44 fmol HPB/g Hb. *A*, peak at 9.887 min, which represents 10 pg of HPB released from 1.42 g of Hb. *B*, peak at 9.883 min, which represents 100 pg of HPB-$d_2$ internal standard added to the Hb sample before hydrolysis.

## Results

The analytical method used in this study is based on a previously published method (5). To shorten analysis time and to increase recovery of HPB, two modifications were made to the existing method: (*a*) a multiple, organic solvent extraction of HPB released from Hb was replaced with a method using solid-phase extraction and (*b*) a lengthy HPLC clean-up of the derivatized HPB was replaced with a simple two-step organic extraction derived from work with other aromatic amines (6). An analytical method using the first modification was recently published (7). Our method also differs from previously approaches in that Ficoll gradient tubes were used to collect RBCs

*Table 1* Within-sample reproducibility for multiple, same-day analysis of aliquots of blood from two donors: HPB released by hydrolysis from Hb

| Sample | No. of aliquots | Hb (g/sample) | Mean ± SD fmol HPB/g Hb | % CV |
|---|---|---|---|---|
| Donor 1 | 8 | 1.80 | 65 ± 10 | 16% |
| Donor 2 | 4 | 1.14 | 434 ± 15 | 4% |

*Table 2* Within-sample reproducibility for the blind duplicate analysis of five human blood samples

| Sample no. | Hb (g/10 ml) | HPB-Hb adduct level | | % CV |
|---|---|---|---|---|
| | | fmol/g Hb | Mean ± SD | |
| 1 | 1.23 | 75 | | |
| 4 | 1.19 | 61 | 68 ± 10 | 15% |
| 2 | 1.50 | 51 | | |
| 10 | 1.34 | 37 | 44 ± 10 | 22% |
| 3 | 1.37 | 47 | | |
| 7 | 1.18 | 52 | 49 ± 3 | 7% |
| 5 | 1.04 | 45 | | |
| 9 | 1.24 | 35 | 40 ± 7 | 17% |
| 8 | 1.21 | 32 | | |
| 6 | 1.42 | 23 | 27 ± 7 | 25% |

in six participating centers. These tubes were designed to collect WBCs and plasma; RBCs have generally been discarded after separation. Thus, it was important to show that the polysucrose-sodium metrizoate Ficoll medium does not interfere with isolation, derivatization, or analysis of HPB.

**Within-Sample Reproducibility for HPB-Hb Adduct Levels.** The modified method was first tested for within-sample reproducibility in two pooled blood samples prepared from the blood from two donors. One pooled sample was divided into four, and the other was divided into eight equal aliquots. As shown in Table 1, within-sample reproducibilities for analysis of these samples, expressed as % CV, were 4 and 16%, respectively.

**Reproducibility of Blind Duplicate Analysis of HPB-Hb Adducts in Blood.** To test the reproducibility of duplicate analyses using the method, blood samples from five other blood donors were divided into two equal aliquots and numbered at random from 1 to 10 by a colleague from outside the laboratory. Hb was isolated from the 10 samples, and HPB was hydrolyzed and analyzed using the method outlined above. Sample identification codes were broken only when all results were available. Intraindividual reproducibility, expressed as % CV, ranged from 7 to 25% for the five samples with a mean of 17% (Table 2).

**Day-to-Day Reproducibility of an Analytical Control Hb Sample.** To control the quality of results over the 4-month analysis period, a large pooled Hb sample was prepared. The sample was mixed well and divided into 40 identical aliquots that were frozen at −80°C. One or more aliquots of this analytical control Hb sample were included with each batch of about eight samples analyzed. Fig. 2 shows the variation in the levels determined for these aliquots over the 4-month analysis period. The level in the analytical control Hb sample ranged from 150 to 198 fmol HPB/g Hb; the average level was 178 ± 16 fmol HPB/g Hb (% CV = 17%). On 4 separate days, three aliquots were analyzed on the same day. Same-day reproducibility for analysis of the control Hb sample, expressed as % CV, ranged from 4 to 10%.

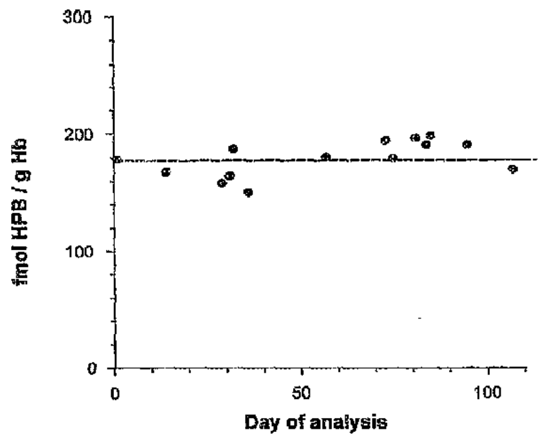


*Fig. 2.* Day-to-day reproducibility over a 4-month period of HPB-Hb levels in aliquots of an analytical control Hb sample.

*Table 3* Mean HPB-Hb adduct levels by sex and smoking status

| | n | HPB-Hb (fmol/g Hb) | | P[a] |
|---|---|---|---|---|
| | | Range | Mean ± SD | |
| Sex | | | | |
| Male | 14 | (12–38) | 22 ± 9 | 0.50 |
| Female | 56 | (9–67) | 21 ± 10 | |
| Smoking status | | | | |
| Smokers | 18 | (9–67) | 26 ± 12 | 0.02 |
| Never-smokers[b] | 52 | (9–38) | 19 ± 8 | |

[a] Kruskal-Wallis rank sum test.
[b] Seven subjects had HPB-Hb adduct levels below the limit of detection (9 fmol HPB/g Hb).

**HPB-Hb Adducts in Study Subjects.** The overall mean HPB-Hb adduct level in the 70 subjects in the study was 21 ± 10 fmol HPB/g Hb (range, 9–67 fmol HPB/g Hb). HPB-Hb adduct levels were significantly higher in smokers than in never-smokers (Table 3). No difference was found between sexes. No difference was seen between current smokers (10 subjects, mean adduct level = 24 ± 7 fmol HPB/g Hb) and ex-smokers (8 subjects, mean adduct level = 29 ± 17 fmol HPB/g Hb). Results of analyses conducted on log-transformed HPB-Hb adduct values yielded virtually identical results.

## Discussion

The purpose of this study was to determine whether HPB-Hb adducts in the blood could be useful to measure exposure to tobacco smoke in never-smokers and ever-smokers. In particular, it was hypothesized that this approach would be useful to integrate low-level exposure from tobacco smoke from several months in the past. A published method (5) was modified to permit simultaneous collection of plasma, WBCs, and RBCs at sites distant from the laboratory of analysis and to allow analysis of at least eight samples per week.

In previously published methods for analysis of HPB-Hb adducts (5, 8), Hb was isolated from fresh RBCs just prior to analysis. However, in multinational, multicenter molecular epidemiology studies, a simple and reliable method is needed

whereby blood can be collected; separated into plasma, WBCs, and RBC; and stored frozen over a period of several months. Ficoll tubes have primarily been used for the isolation of WBCs, in which the plasma and RBCs often are discarded. The results of our study indicate that Hb can be collected at the same time as plasma and WBCs and that the Ficoll layer of polysucrose and sodium metrizoate in the collection tube does not interfere with our GC/MS method or quantification of the level of adducts. Use of Ficoll tubes for isolation of RBCs, WBCs, and plasma facilitates collection of these cell types from the same subjects. As techniques become available and our understanding of the relationship between inter- and intracellular changes increases, it will be important to have different cell types from the same individual. Moreover, use of a Ficoll tube permits some standardization in collection and isolation of cell types between geographically distant study sites.

Carmella *et al.* (5) first used Hb adducts of HPB, a metabolite of the tobacco-specific nitrosamines $N'$-nitrosonornicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, as markers of exposure to tobacco smoke in humans. Elevated levels of HPB-Hb adducts in 40 smokers (80 ± 190 fmol HPB/g Hb; range, 4.8–1160) and 21 nonsmokers (29 ± 26 fmol/g Hb; range, 8.5–95) were clearly related to exposure to tobacco smoke. However, for both smokers and nonsmokers, a large heterogeneity was observed, based on the range of HPB levels. This study was later extended to 100 smokers (163 fmol HPB/g Hb) and 37 nonsmokers (68 fmol HPB/g Hb; Ref. 9). A similar study (10) on smokers (69 ± 44 fmol/g Hb) and nonsmokers (34 ± 16 fmol/g Hb) and a recent study by the same group (8) in pregnant smoking (55 ± 46 fmol/g Hb) and nonsmoking (27 ± 35 fmol/g Hb) women gave similar results. Smoking status in these articles was defined as "smoker" and "nonsmoker," and it was assumed that "current" smoking status was assessed at the time of interview.

The mean levels of HPB adducts reported in this study in both smokers (26 ± 12 fmol HPB/g Hb) and never-smokers (19 ± 8 fmol HPB/g Hb) were lower than those among smokers and nonsmokers in previous studies (5, 8, 9, 10). This may be because most participants in the study were hospital patients and may have smoked less or have been exposed to less tobacco smoke in the period just before blood collection than participants in previous studies. In addition, many ever-smokers were long-time former smokers. In previous studies, it appears that smokers were generally current smokers, whereas nonsmokers may have included former smokers or recent quitters, so that results may not be fully comparable. When HPB-Hb adduct levels were measured in blood samples taken at random from the general population to validate our method (Table 2), results were comparable to those reported in previous studies.

Within-sample, interindividual, and day-to-day reproducibilities were shown to be acceptable for the modified methodology and HPB-Hb adduct levels were shown to be significantly higher ($P = 0.01$) in ever-smokers than in never-smokers. These results suggest that the level of HPB-Hb adducts, measured using our method and modified to facilitate use in multicenter studies, can be a useful biomarker of exposure to tobacco smoke.

## Acknowledgments

We thank Stephen S. Hecht (University of Minnesota Cancer Center, Minneapolis, MN) for the [4,4-$D_2$]-HPB and nonlabeled HPB standards used in this study; Arno Helbringer (Center de Transfusion Sanguine, Lyon, France) for providing blood samples used in development and validation of the analytical method; and Nadejda Jourenkova for her assistance with sample management.

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